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Role of Cytomegalovirus Reinfection in Acute Rejection and CMV Disease After Renal Transplantation

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1. Introduction

Renal transplantation is a most valuable treatment for patients with end-stage renal disease as it offers improved survival and quality-of-life benefits compared with dialysis (Evans, Manninen et al. 1985; Port, Wolfe et al. 1993). However, there has been no satisfactory increase in long-term graft survival despite significant advances in the field of renal transplantation (Meier-Kriesche, Schold et al. 2004). Long-term graft failure is generally due to death despite a functioning graft, chronic rejection, or recurrent kidney disease (Valente, Hariharan et al. 1997). Among these, chronic rejection is the most important cause of long-term graft failure (Jindal and Hariharan 1999). Graft failure owing to chronic rejection is a common reason for retransplantation, and the most important predictor of chronic rejection is a previous episode of acute rejection (Almond, Matas et al. 1993; Hariharan, Alexander et al. 1996; Cosio, Pelletier et al. 1997). Clinical acute rejection within the first year after transplantation has been reported to have a detrimental effect on long-term graft survival (Hariharan, Johnson et al. 2000). The projected half-life for cadaveric transplants in patients who did not have an episode of clinical acute rejection in the first year after transplantation was 17.9 years in 1995, compared with that of 8.8 years for patients who had an episode of clinical acute rejection (Hariharan, Johnson et al. 2000). This reduction in the relative risk of graft failure was significant among patients without acute rejection, whereas no reduction in the relative risk of graft failure was observed among those with acute rejection. Thus, the main issue asociated with renal transplantation is the suppression of allograft rejection.

Cytomegalovirus (CMV) infection, which is the most common infection following renal transplanation, continues to be a potential contributor to graft loss and a cause of severe mortality and morbidity. Several studies have suggested that CMV infection can lead to allograft rejection (Lautenschlager, Soots et al. 1997; Humar, Gillingham et al. 1999; McLaughlin, Wu et al. 2002; Meier-Kriesche, Schold et al. 2004; Nett, Heisey et al. 2004). Although the role of CMV infection in acute rejection after renal transplantation remains controversial, many reports have demonstrated that CMV serostatus, as defined by conventional classifications, influences clinical outcome in renal transplantation (Humar, Gillingham et al. 1999; McLaughlin, Wu et al. 2002). The combination of CMV-seronegative recipients (R-) with CMV-seropositive donors (D+) led to the highest risk of CMV disease.

Historically, concern has focused on avoiding CMV infection in D+/R- settings. However, some transplant recipients in the D+/R+ group experience severe CMV disease and/or acute rejection despite their pre-existing immunity. For example, in D+R+ cases, approximately 20% of recipients experienced CMV disease in the absence of any prophylaxis (Sagedal, Nordal et al. 2000). Analysis of the data from the United States Renal Data System and United Network of Organ Sharing revealed that the D+/R+, not the D+/R- group, had the worst graft and patient survival by 3 years (Schnitzler, Woodward et al. 1997; Schnitzler, Woodward et al. 1997). This may reflect the prevalence of multiple CMV virotypes, and that D+/R+ recipients may have double exposure to different CMV strains.

This article addresses the impact of CMV reinfection with different CMV strains on the clinical course after renal transplantation.

2. Background

2.1 Cytomegalovirus virology

CMV is the fifth member of the 8 known human herpes viruses (HHVs). HHVs are classified into three subgroups, alpha herpesvirinae, beta herpesvirinae and gamma herpesvirinae. CMV, a member of the beta herpes virus family together with HHV-6 and HHV-7, is a widespread opportunistic pathogen. Primary CMV infection usually occurs during the first decades of life. CMV is transmitted via salvia, body fluids, cells and tissues (Egli, Binggeli et al. 2007). Primary infection is followed by a latent infection that can persist throughout the entire life of the host. The principal reservoirs of latent CMV are the white blood cells and CD13-positive cells (Larsson, Soderberg-Naucler et al. 1998) and the latent virus has been detected in most tissues in the body. CMV can infect most renal cell types, including glomerular, tubular, and endothelial cells (Heieren, Kim et al. 1988; Heieren, van der Woude et al. 1988; Ustinov, Loginov et al. 1991).

CMV is a DNA virus containing 230-kb double-strand DNA. CMV has a typical herpes virion structure consisting of viral DNA, capsid, tegument and envelope. The envelope contains structural proteins including glycoproteins. The glycoproteins are used for cellular entry by the virus. CMV initially tethers itself to cell-surface heparan sulfate proteoglycans (HSPGs) via viral envelope glycoproteins (Kari and Gehrz 1992; Compton, Nowlin et al. 1993), although HSPGs alone are not sufficient to mediate viral entry. The ability of CMV to enter a wide variety of cell types indicates multiple receptors for entry, and many cell-surface components have been identified as virus receptors. It is reported that CMV uses epidermal growth factor receptor (EGFR), platelet-derived growth factor-α receptor (PDGFR-α) and cellular integrins for cellular entry (Wang, Huong et al. 2003; Feire, Koss et al. 2004; Soroceanu, Akhavan et al. 2008), although these findings remain controversial (Isaacson, Feire et al. 2007). The entry of CMV into host cells requires interactions between cellular and viral molecules, and glycoproteins. Pretreatment of CMV with glycoprotein-specific antibodies could disrupt interaction between CMV and cellular molecules (Wang, Huang et al. 2005; Soroceanu, Akhavan et al. 2008).

The complete genome of the laboratory strain of CMV AD169 has been sequenced (Chee, Bankier et al. 1990), however, genetic differences among CMV strains have been observed in multiple genes. Those polymorphisms may be implicated in immunopathogenesis as well as CMV strain-specific behavior and cell tropism.

2.2 Glycoproteins of Cytomegalovirus

The initial events associated with CMV infection require interactions between the host cell-surface molecules and the CMV envelope. The early steps of virion attachment, fusion and penetration of the host cell have been assumed to be functions of the viral envelope glycoproteins. To date, at least 57 potential glycoproteins are known to be encoded by the laboratory strain of CMV (AD169), and several envelope glycoproteins have been characterized (Chee, Bankier et al. 1990; Britt and Mach 1996; Cha, Tom et al. 1996). These glycoproteins associate in high molecular weight complexes and the mature complexes are referred to as glycoprotein complex I (gC-I), glycoprotein complex II (gC-II) and glycoprotein complex III (gC-III). The genes encoding glycoproteins often show genetic polymorphism. Because the envelope glycoproteins elicit strong host immune responses, including the production of neutralizing antibodies, understanding of the genetic polymorphism of the glycoproteins will have an impact on strategies to protect against CMV infection as well as clinical studies.

2.2.1 Glycoprotein H (gH)

The disulphide-bond tripartite gC-III envelope complex consists of gH, gL and gO (Huber and Compton 1999). Glycoprotein H is one of the immunologically dominant glycoproteins in the CMV envelope, and is encoded by open reading frame (ORF) unique long (UL) region 75 (Pachl, Probert et al. 1989). The gH gene of the AD169 strain is 2,229 nucleotides long and that of the Towne strain is 2,226 in length. The gH product of AD169 UL75 comprises 743 amino acids (aa) and that of Towne is 742 aa, with a deletion of proline 36 (Pachl, Probert et al. 1989; Britt and Mach 1996). Although the UL75 is highly conserved among multiple CMV strains, sequence variations were found in the first 37 aa (Chou 1992). Based on the sequence analysis of UL75 from multiple strains, it was estimated that CMV gH has two genotypes (Chou 1992). gH mediates viral/host cell membrane fusion in the initial step of infectivity, and monoclonal anti-gH antibodies can inhibit virus infectivity (Keay and Baldwin 1991). Antibodies against gH can be detected after natural infection with CMV (Rasmussen, Matkin et al. 1991). Anti-CMV gH antibodies exhibit virus neutralizing activity and gH is considered a major antigen for the humoral immune response (Urban, Klein et al. 1996). A linear antibody binding site is located within the amino-terminal region of gH (aa 34-43), which shows sequence heterogeneity between the AD169 and Towne strains (Urban, Britt et al. 1992). This heterogeneity is characterized by the deletion of a proline residue at position 36 and the substitution of lysine for histidine at position 37 in the Towne strain as compared with the AD169 strain. This antibody binding site is recognized as strain-specific epitope(Urban, Britt et al. 1992), and the heterogeneity influences CMV susceptibility to host neutralizing antibodies. A recent report on congenital CMV infection has provided clear evidence that exposure to CMV with a different genotype causes congenital infection, even in seropositive mothers (Boppana, Rivera et al. 2001).

2.2.2 Glycoprotein B (gB)

Glycoprotein B, a component of the envelope complex gC-I, is the most abundant glycoprotein in the CMV envelope. gB is one of the most highly conserved components among all members of the herpesvirus family. It is encoded by UL55 and exhibits genetic

polymorphism. The 906 aa polypeptide of the AD169 strain gB is cleaved at position 460 by a cellular endoprotease. Nucleotide and peptide sequence analysis revealed that variations were most frequent between positions 448 and 480, which includes the cleavage site (Chou and Dennison 1991). Restriction enzyme analysis has identified four main gB groups (gB-1, gB-2, gB-3 and gB-4). While variations were found in gB, substantial conservation of the peptide sequence is observed in this region. The closely regulated variations in gB may suggest its important role in the viral life cycle (Chou and Dennison 1991). Although little is known of the effect of gB variations on biologic function, genetic variations have been used for epidemiologic purpose.

gB has a role in binding to cell surface receptors, and neutralizing gB-specific antibodies can inhibit the binding (Ohizumi, Suzuki et al. 1992). CMV interacts with cellular integrins, EGFR and PDGFR-α through gB, and these compounds are considered as potential cellular receptors of CMV (Wang, Huong et al. 2003; Feire, Koss et al. 2004; Soroceanu, Akhavan et al. 2008).

The antigenicity of gB has been well studied and linear neutralizing and non-neutralizing antibody-binding sites have been defined (Britt and Mach 1996). The antigen domain 1 (AD1), which is located between positions 560 and 640 of gB, is a major neutralizing epitope (Schoppel, Hassfurther et al. 1996) and is the most highly conserved region among viral strains. The second antibody binding site on gB is the antigen domain 2 (AD2), which is located between aa 28 and 84 of gB (Meyer, Sundqvist et al. 1992). Within the AD2 domain, two antigenic sites have been identified. Site I is located between aa 68 and 77, and this region is conserved among CMV wild-type strains and is the target of neutralizing antibodies. Site II, another binding sequence in the AD2, is located between aa 50 and 54. Site II binds non-neutralizing antibodies and is strain-specific (Meyer, Sundqvist et al. 1992).

2.2.3 Glycoprotein N (gN)

Glycoprotein N is a component of the envelope complex gC-II (Mach, Kropff et al. 2000; Dal Monte, Pignatelli et al. 2001). gN has been identified as one of the major antigens together with gH and gB (Shimamura, Mach et al. 2006). It is encoded by the ORF UL73, and antibodies against gN neutralize virus infectivity. The immunogenic gC-II is also the major heparin binding complex (Kari and Gehrz 1992).

UL73 has four main genomic variants, denoted gN-1, gN-2, gN-3 and gN-4 (Pignatelli, Dal Monte et al. 2001). The worldwide geographical distribution of identified variants of the gN was investigated and it was found that the variants do not necessarily exhibit the same frequency distribution (Pignatelli, Dal Monte et al. 2003). The gN genomic variants are related to the immunopathogenesis of CMV in immunocompromised hosts and in congenitally infected infants (Pignatelli, Dal Monte et al. 2003; Pignatelli, Rossini et al. 2003).

2.2.4 Other Glycoproteins

Other than gH, gB and gN, the large CMV genome encodes many additional glycoproteins. UL100 encodes glycoprotein M (gM), which, together with gN, is a component of gC-II. gM is essential for viral replication (Hobom, Brune et al. 2000), and seems to be highly

conserved (Lehner, Stamminger et al. 1991). It was shown that most sera failed to react with either gM or gN alone (Mach, Kropff et al. 2000). Virus neutralizing antibodies were shown to be directed at the gN component of the gM–gN complex.

In addition to gH, the gC-III envelope complex contains glycoprotein O (gO) and glycoprotein L (gL) (Huber and Compton 1999). gO is encoded by the UL74 ORF. The sequence analysis of UL74 showed a high degree of variability at the N-terminal end (Paterson, Dyer et al. 2002). The analysis of clinical isolates identified four major phylogenetic groups, denoted gO-1, gO-2, gO-3 and gO-4 (Mattick, Dewin et al. 2004). gL is encoded by the UL115 ORF. Four major phylogenetic groups were again identified and denoted gL-1, gL-2, gL-3 and gL-4 (Rasmussen, Geissler et al. 2002). gL is essential for the transport of the gH glycoprotein to the cell surface (Kaye, Gompels et al. 1992; Spaete, Perot et al. 1993).

The large number of gH-gO-gL combinations suggests that gC-III has an immunological potential, and has implications for viral tropism and spread (Rasmussen, Geissler et al. 2002).

2.3 CMV genpotype and renal transplantation

Transplantation in a D+/R+ setting is usually accompanied by multiple CMV strains in recipients after transplantation (Manuel, Pang et al. 2009), with mixtures of gB and gH genptypes were commonly observed in organ transplant recipients (Zhou, Fan et al. 2007). As CMV displays genetic polymorphism among glycoproteins thought to be implicated in tissue tropism and immunopathogenesis, an association betweenf glycoprotein genotype with CMV infection in organ transplantation has been reported (Woo, Lo et al. 1997) (Humar, Kumar et al. 2003; Retiere, Lesimple et al. 2003; Coaquette, Bourgeois et al. 2004; Rossini, Pignatelli et al. 2005; Zhou, Fan et al. 2007; Manuel, Asberg et al. 2009). It is known that gB plays an important role in virus entry and is also a target of neutralizing antibodies (Cranage, Kouzarides et al. 1986; Ohizumi, Suzuki et al. 1992; Navarro, Paz et al. 1993; Hopkins, Fiander et al. 1996; Lantto, Fletcher et al. 2003). Therefore, many studies have attempted to find a correlation between gB genotype and the occurrence of CMV infection or disease in organ transplant recipients. In a study involving 50 transplant recipients, the gB genotype was found not to effect viral load or clinical response to therapy (Humar, Kumar et al. 2003). Although another study also found no significant differences among gB genotypes with regard to the development of symptomatic disease, acute graft rejection, or CMV load, immunocompromised patients infected with multiple gB genotypes showed a progression to CMV disease, had an increased rate of graft rejection and had higher CMV loads (Coaquette, Bourgeois et al. 2004). Recent large, prospective cohort studies of organ transplantation have shown mixed infection to be associated with higher viral loads and delayed virologic clearance according on the basis of gB distribution analysis (Manuel, Asberg et al. 2009). Mixed genotype infection was more likely when both donor and recipient were CMV seropositive.

In addition to gB, associations between gN genotype and clinical features in organ transplant recipients were investigated (Rossini, Pignatelli et al. 2005). This study involving 74 solid organ transplant recipients showed a difference in virulance between those with the gN-1 and gN-4 strains. The gN-4 strain was associated with higher levels of antigenemia-

positive cells. However, in this study, no mixed gentype infection was found and only 19 of the 74 recipients underwent kidney transplantation. Thus the influence of infection with multiple gN genotypes in cases of renal transplantation remains unclear.

These studies of glycoprotein genotypes may have some implications for the immunity of recipients as well as viral pathogenesis. gB has been implicated in host cell entry, cell-to-cell transmission of virus and fusion of infected cells, and is an important target for humoral and cellular immune responses (Cranage, Kouzarides et al. 1986; Navarro, Paz et al. 1993; Hopkins, Fiander et al. 1996; Lantto, Fletcher et al. 2003). Although polymorphisms in the cleavage site of the gB gene allow classification into of 4 distinct genotypes, the sequence of the antigenic site is well conserved (Roy, Grundy et al. 1993). The linear antibody binding sites include AD-1 and AD-2, which induce neutralizing antibodies and are highly conserved (Roy, Grundy et al. 1993). It seems that gB genotypes based on the cleavage site don't affect CMV infection in transplant recipients (Humar, Kumar et al. 2003). However, mixed gB genotype infections are associated with severe clinical manifestations (Coaquette, Bourgeois et al. 2004; Manuel, Asberg et al. 2009). In contrast to gB, the linear neutralizing antibody binding sites on gH and gN exhibit variations and form strain-specific epitopes (Urban, Britt et al. 1992; Burkhardt, Himmelein et al. 2009). These variations in the antibody binding epitopes of glycoproteins influence viral infection as they allow the virus to evade humoral immune responses.

3. CMV gH strain-specific antibody epidemiology of cytomegalovirus

3.1 ELISA for glycoproteins

The induction of an effective antibody response against CMV is an important defense mechanism as it is capable of neutralizing infectious viruses. An analysis of transplant patients revealed that during primary infection strain-specific and strain-common antibodies are produced asynchronously (Klein, Schoppel et al. 1999). The strain-specific neutralizing antibodies are induced during infection with CMV. Because CMV can persist throughout the entire life of the host after primary infection, we hypothesized that detection of strain-specific antibodies can be used to identify the CMV strain latent in the host. To evaluate CMV glycoprotein strain-specific antibodies, we analyzed for strain-specific immunoglobulin G (IgG) antibodies against the polymorphic epitopes on the envelope gH glycoproteins of CMV by means of an enzyme-linked immunosorbent assay (ELISA) method (Ishibashi, Tokumoto et al. 2007; Ishibashi, Tokumoto et al. 2008).

CMV strain-specific antibody responses were determined on the basis of polymorphisms in the antibody binding epitopes within gH between the 2 prototypical laboratory strains of CMV, AD169 and Towne. In addition to gH, we employed AD2 site I, which is conserved among CMV isolates and is the target of neutralizing antibodies, as well as AD2 site II, which binds non-neutralizing antibodies and is strain specific, for the detection of antibodies against gB (Figure 1).

3.2 Strain-specific seroepidemiology of CMV

The prevalence of CMV seropositivity varies around the world with seroprevalence ranging from 45 to 100% (Cannon, Schmid et al. 2010). For example, the CMV seroprevalence is

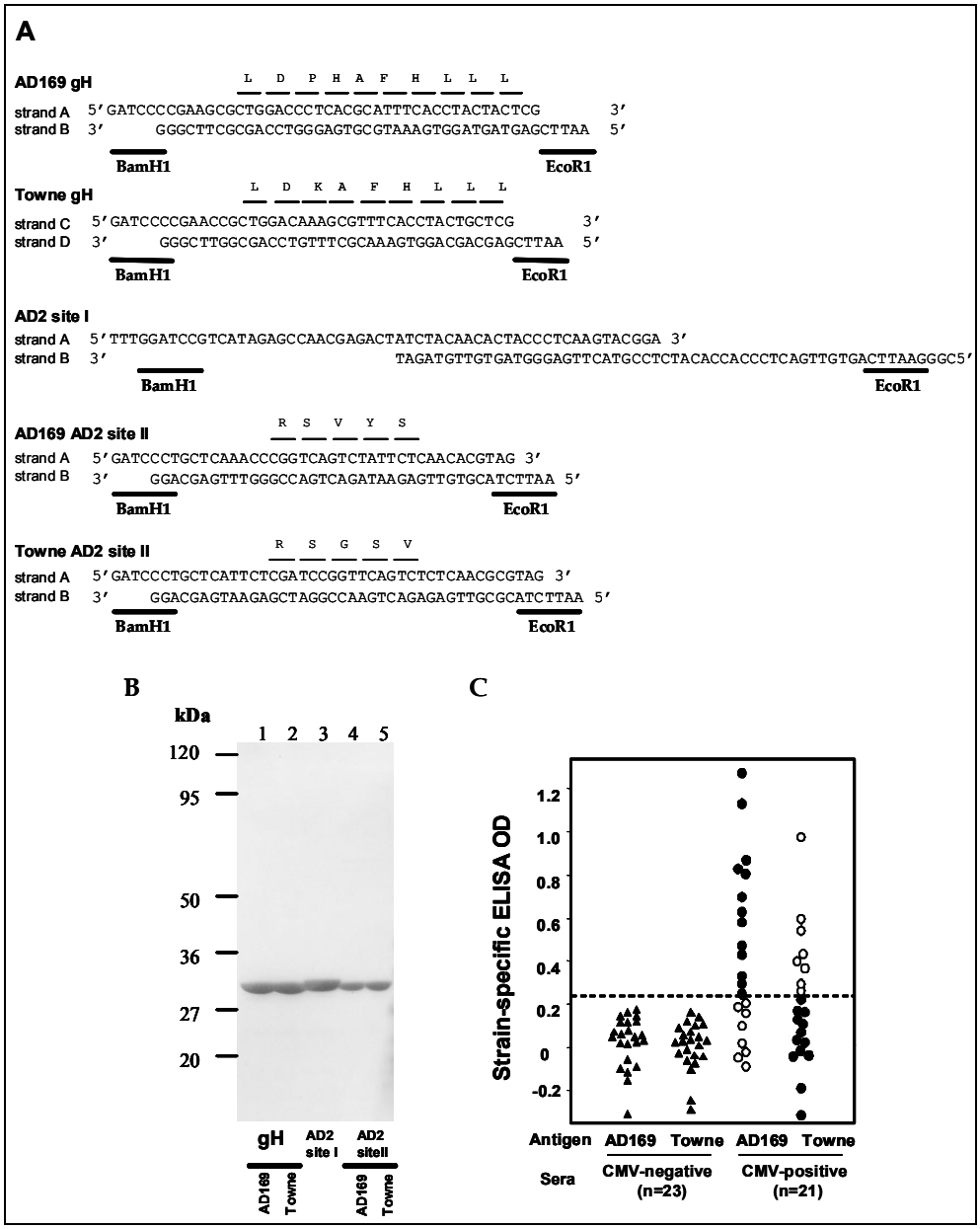

Fig. 1. Cloning and expression of recombinant antigens of CMV glycoproteins for ELISA. **A**, Oligonucleotides containing CMV gH epitopes, gB AD2 site I and site II from the AD169 and Towne strains, were used for the expression of gH epitopes as GST fusion proteins. DNA fragments encoding the epitopes were prepared by annealing two synthetic oligonucleotides. The DNA strand for the AD2 site I region was synthesized by Taq DNA

polymerase after annealing the two oligonucleotides. Each cassette has BamHI and EcoRI sites at the ends for cloning. These DNA cassettes were cloned into the *EcoRI* and *BamHI* sites of expression vector pGEX-5x. **B**, SDS-PAGE of the purified GST fusion proteins containing the AD169-gH (lane 1), Towne-gH (lane 2), AD2 site I epitope (lane 3), AD169-AD2 site II (lane 4) and Towne-AD2 site II epitopes (lane 5). Each epitope was expressed in *E. coli* strain DH5α as a fusion protein with GST, and purified using GSTrap FF (Amersham Bioscience). Size standards are shown on the left side of the gel. **C**, ELISA using purified GST fusion proteins containing AD169- and Towne-specific gH epitopes. Reactivity of CMV-seronegative sera (closed triangles) and CMV-seropositive sera reacting with the gH epitopes specific to AD169 (closed circles) and Towne (open circles) were plotted. A dashed horizontal line indicates the cut-off OD. Optical density (OD) values specific to each strain-specific gH antigen were obtained by subtracting the OD values for GST. An arbitrary cutoff for the ELISA (OD=0.25) was defined as the mean plus two standard deviations (SD) of OD values obtained from a panel of 23 healthy CMV seronegative volunteers. Figures were modified from our recent papers (Ishibashi, Tokumoto et al. 2007; Ishibashi, Tokumoto et al. 2008).

93.8% in Japan, 86.7% in Chile (Lagasse, Dhooge et al. 2000), 82.5% in the United States (Fowler, Stagno et al. 2003) and 49.5% in France (Lepage, Leroyer et al. 2011). CMV antibodies reflect prior exposure and existing immunity. However, it has been reported that the protection conferred by pre-existing immunity is limited because of the strain-dependence of the immune responses(Boppana, Rivera et al. 2001). CMV exists as a variety of different strains according to the genotype of its envelope glycoproteins. The analysis of CMV reinfection or of mixed infections has become increasingly important. Reinfection may occur when a subsequent CMV infection evades the pre-existing humoral immunity of the host. In this study, which was approved by the institutional ethics committee, blood samples were obtained from a total of 352 subjects (aged 15 to 75), consisting of healthy volunteers and consecutive potential donors and recipients for renal transplantation. We employed ELISA using GST-fusion proteins containing the strain-specific gH epitopes from AD169 and Towne strains as well as gB AD2 site I and AD2 site II to detect preexisting strain-specific antibodies in transplant recipients and healthy blood donors. The distribution of antibody responses against glycoproteins is summarized in Figure 2.

A panel of sera obtained from 352 blood donors was evaluated using the GST fusion proteins. Among the 255 serum samples with antibodies against gH and/or gB, 207(81.2%) were reactive with the gH ELISA and 178(69.8%) with the gB AD2 site I ELISA, with 132 samples reactive with both gB and gH. The CMV seropositive rate was lower in subjects aged in their teens (50%) and 20s (62%), and the rate increased significantly with increases in age, reaching 80-90% in subjects aged 30 years or over. Strain-specific antibody responses among the 207 gH seropositive samples showed that 44 samples were reactive with the gH of both AD169 and Towne. Of the 44 donors whose serum contained antibodies against both AD169 and Towne, 27 (61%) were aged 50 years or over (Figure 2B). This dual-positive rate was significantly higher than that for donors under 50 years ($p<0.01$). This result indicates that organ transplantation from older donors to younger recipients; for example, from a father or mother to one of their children, as is common in living-related transplantation, can increase the risk of reinfection with CMV.

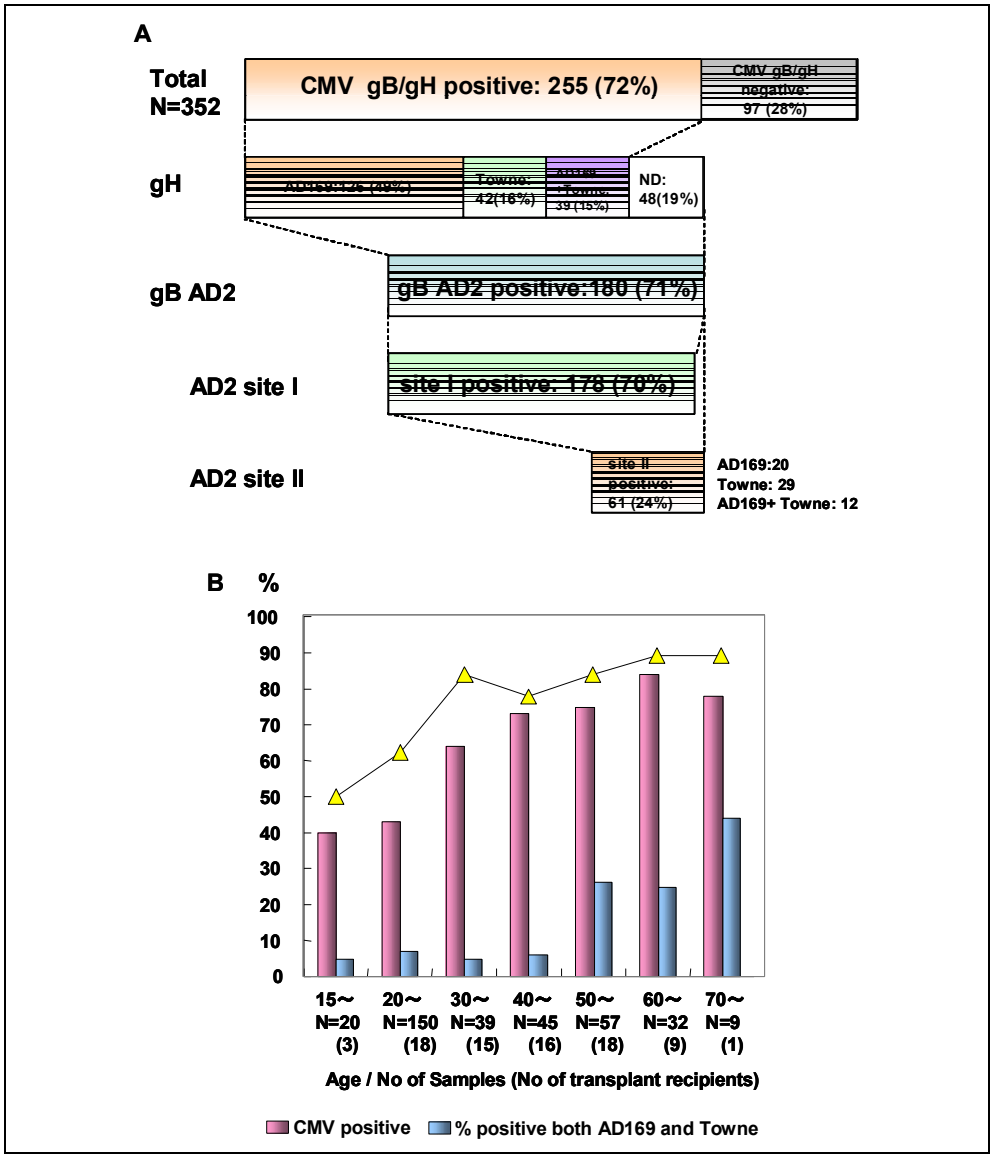

Fig. 2. Distribution of gH and gB-specific antibodies and their seroprevalence.
A: Summary of the number and distribution of serum samples according to antibody responses against gH epitopes and gB AD2.
B: Seroprevalence of CMV. Closed yellow triangles indicate CMV serostatus analyzed using a conventional ELISA kit. Rate of positive antibodies against gH and rate of positive strain-specific antibodies against both AD169 and Towne strains according to age. Age group and number of serum samples are shown on the horizontal axis. Figures were modified from our recent paper (Ishibashi, Tokumoto et al. 2008).

3.3 Association of HLA-DR with strain-specific antibodies

Several reports have described the association between the occurrence of viral infection and disease and the presence of certain HLA antigens in the host (Stewart, Kelsall et al. 1981; Baldwin, Claas et al. 1983; Roenhorst, Tegzess et al. 1985; Kraat, Christiaans et al. 1994; Varga, Rajczy et al. 2008). Many HLA antigens, including HLA-A2, HLA-A24, HLA-A32, HLA-B52, HLA-Bw4, HLA-DR6, HLA-DR11, HLA-DR15 and HLA-DQ3, were reported to increase the risk of CMV infection (Roenhorst, Tegzess et al. 1985; Chen, Rocha et al. 2001; Hodge, Boivin et al. 2004; Fan, Meng et al. 2006), although there were some conflict among the results (Gomez, Aguado et al. 1993). Major histocompatibility complex (MHC) molecules are critical for the presentation of antigens. The association between HLA alleles and CMV infection may be due to the differential presentation of CMV peptides by HLA molecules and/or the differential recognition by host lymphocytes. Studies in animals showed a genetic susceptibility to CMV infection which is controlled by the MHC (Chalmer, Mackenzie et al. 1977; Grundy, Mackenzie et al. 1981). Although the immune responses to CMV infection could be linked to CMV infection, polymorphism of CMV antigenic proteins may constitute an immune evasion mechanism. The differential presentation of polymorphic gB or Immediate Early-1 peptide by HLA molecules was suggested by the data from renal transplant recipients (Retiere, Lesimple et al. 2003). Another possible association between HLA and CMV infection is in the production of antibodies. It has been reported that deficiencies in the production of neutralizing antibodies against CMV gB in certain HLA types may lead to increased susceptibility (Wada, Mizuno et al. 1997). In this report, subjects with HLA-DR9 had a higher positive rate against CMV gB and subjects with HLA-DR15 had a lower positive rate against gB. Production of antibodies against another major epitope of CMV, gH, may correlate with certain HLA types. We investigated HLA-DR type and strain-specific antibody responses against gH in potential donors and recipients for renal transplantation (Ishibashi, Tokumoto et al. 2009). Our results showed that subjects with HLA-DR10 showed a significantly lower response rate against CMV gH and subjects with HLA-DR11 had a lower response rate. It is of interest that there have been some reports that HLA-DR11 alleles are more susceptible to active CMV infection in the case of solid organ transplantation (Retiere, Lesimple et al. 2003; Fan, Meng et al. 2006). However, the percentages of subjects with HLA-DR10 and HLA-DR11 were very small in our study population. Geographical analysis of the distribution of antibodies against CMV glycoproteins and HLA types would be of interest.

4. Differences in adverse events between CMV primary infections and reinfections

4.1 Pattern of infection and clinical impact of CMV

CMV infection and disease are crucial causes of morbidity and mortality among transplant recipients. The term "CMV infection" applies to a condition in which there is evidence of CMV replication whether or not symptoms are present. There are three main patterns of CMV infection, primary infection, reactivation and reinfection (Ljungman, Griffiths et al. 2002). Primary infection is defined as a new-onset infection in recipients who had been found to be seronegative. The recipients acquire CMV from their donors for the first time. If the latent infected recipient's original CMV is reactivated in recipients who were

seropositive, it is understood as reactivation. Reinfection is defined as infection of new CMV strain in recipients who were previously seropositive. Reinfection is diagnosed if the detected CMV strain was different from that of their donor's by using a variety of molecular techniques or inferred if the recipients acquire new antibodies against strain-specific epitopes.

CMV infections in solid organ transplant recipients induce serious direct and indirect consequences. The direct clinical effects of CMV include CMV infection, CMV disease and end-organ diseases; *i.e.,* gastrointestinal disease, hepatitis, retinitis, nephritis, cystitis, myocarditis, encephalitis and pancreatitis. In addition to the directly effects, CMV is associated with graft rejection, allograft dysfunction and failure, cardiovascular complications, and fungal, viral or bacterial superinfection, all of which are known as the "indirect effects" of CMV (Ljungman, Griffiths et al. 2002).

Classically, because of its high rate of CMV primary infection, concern was mainly focused on CMV in D+/R- settings. However, in D+/R+ transplantation, the presence of antibodies against matched CMV gH epitopes influence the outcome of transplantation. More adverse events were observed in the case of reinfection with different CMV strains.

4.2 Classification of transplant pairings according to CMV strain-specific antibody responses

To identify potential CMV reinfections before renal transplantation, genotypes of the CMVs latently infecting donors and recipients should be detected. One of the methods considered for the genotyping of CMV is the detection of CMV DNA directly from blood donors using PCR. However, CMV DNA during the latency phase is seldom detected in healthy blood donors with validated PCR (Roback, Drew et al. 2003). The low copy number of latency infected cells is a major limitation to PCR. It is estimated that 0.004 to 0.12 percent of CMV-positive peripheral blood mononuclear cells harbor 2 to 13 genomes per cell (Slobedman and Mocarski 1999; Soderberg-Naucler, Streblow et al. 2001). The highest detection rate obtained with PCR to date was approximately 39% (Pignatelli, Dal Monte et al. 2006).

Instead of PCR methods, we employed a serologic assay using *E. coli*-expressed strain-specific epitopes (Ishibashi, Tokumoto et al. 2007). This serologic assay can estimate the CMV strain previously and latently infecting the blood donors. From the combination of antibody responses against the strain-specific gH epitopes, the conventional CMV D+/R+ pairings can be classified into two groups. When a recipient receives an organ graft from a donor who has the same strain-specific gH antibody of CMV as the recipient, the pairing is classified as a "matched gH" pairing. The pairings in which the recipients do not have strain-specific gH antibodies matching their donor's are classified as "mismatched gH" pairings. The "mismatched gH" pairings indicate that the recipients can not neutralize the CMV from donors. It is considered that this pairing in a D+/R+ setting can cause CMV reinfection. We distinguished 114 renal transplantation pairings according to the strain-specific responses and analyzed the data (Figure 3). We found differences in the clinical course after transplantation among the D+/R-, matched gH and mismatched gH pairings.

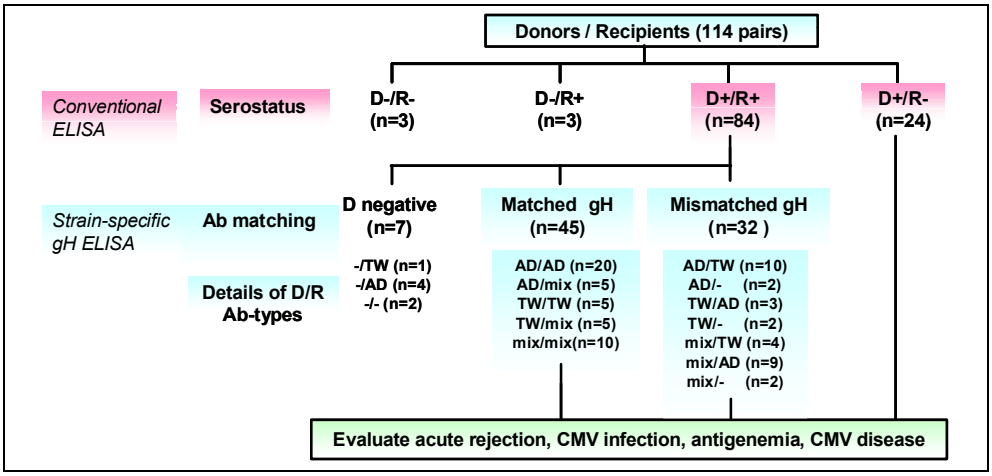

Fig. 3. Classification of transplant pairings according to strain-specific gH antibody responses. Classifications of the donors (D) and recipients (R) according to the conventional CMV serostatus (+ or -) and CMV strain-specific antibodies (Ab) against gH epitopes. Details of the combinations based on strain-specific Ab against gH epitopes (AD: AD169, TW: Towne, mix: both AD169 and Towne, and -: undetectable) are also shown. The 84 D+/R+ pairs were classified into three subgroups, "matched gH", "mismatched gH" and "D-negative". As antibodies against gH were undetectable in 7 donors, they were excluded from the analysis. The figure was slightly modified from our recent paper (Ishibashi, Tokumoto et al. 2007).

4.3 Acute rejection after renal transplantation

In this study, which was approved by the institutional ethics committee, 114 pairs of consecutive donors and recipients undergoing living-related renal transplantation were included. Immunosuppression for recipients consisted of triple-drug therapy (tacrolimus or cyclosporine, mycophenolate mofetile, and predonisolone). Among the 114 transplants, cyclosporin was used in 7 recipients who had chest pain or hyperglycemia. Rejection was suspected when serum creatinine level increased more than 25% from the basal level in the absence of urinary tract obstruction or renal graft artery stenosis. During the follow up after transplantation, the first rejection episode was confirmed histologically by biopsy samples from the grafts.

Five recipients (21%) among the 24 D+/R- pairs and 31 recipients (37%) among the 84 D+/R+ pairs experienced biopsy-proven acute rejection. There was no statistically significant difference in the acute rejection rate between the D+/R- and D+/R+ settings (p=0.14). Among the 27 D+/R+ patients with acute rejection whose matching of strain-specific gH antibodies were known, 17 (53%) did not have matched strain-specific antibodies and was categorized as "mismatched gH"(Figure 4). The rate of acute rejection after renal transplantation was significantly higher in recipients in "mismatched gH" groups than in those of "matched gH" groups (22%; p=0.0051) and of the D+/R- groups (21%;, p=0.014). The average number of days after transplantation to diagnosis of acute rejection was 25 days for all cases with acute rejection, and there were no statistical differences in incubation period for acute rejection among the three groups.

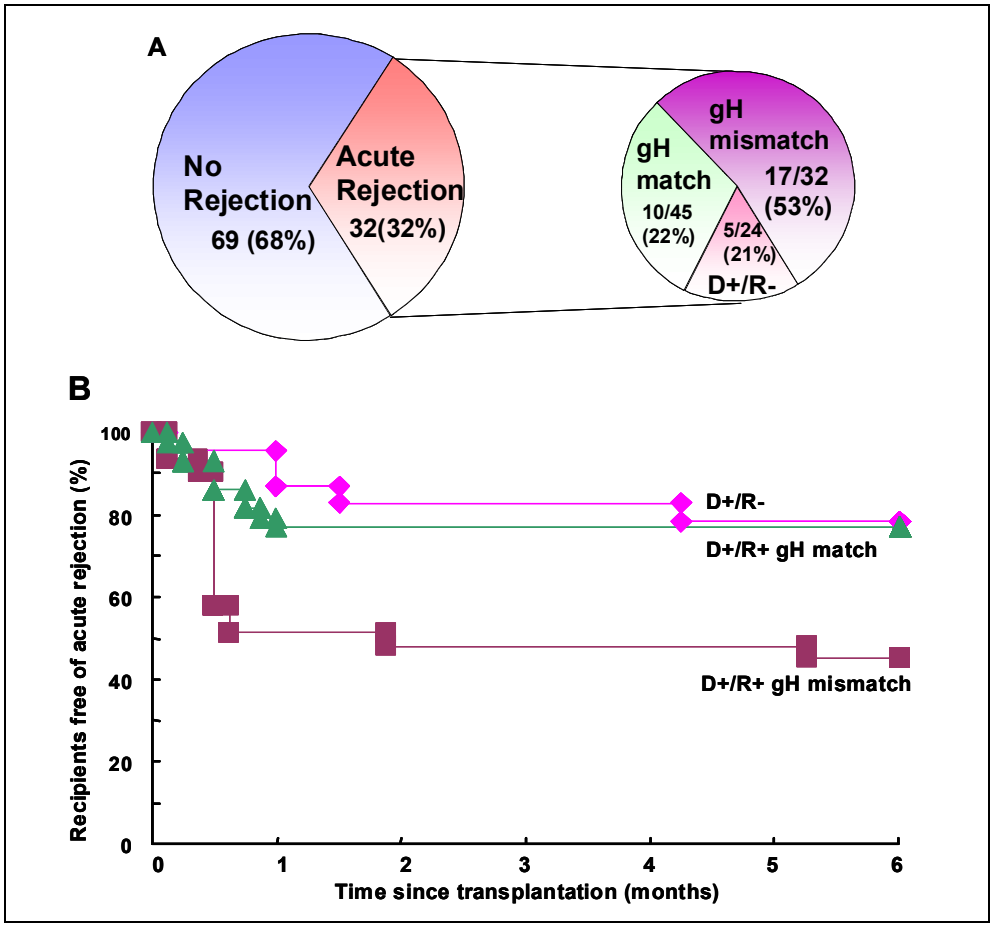

Fig. 4. Incidences of acute rejection.
A, Among the 101 transplant recipients with the three types of settings, the D+/R- (n=24), D+/R+ matched gH (n=45) and D+/R+ mismatched gH (n=32) groups, 32 (32%) experienced biopsy-proven acute rejection after transplantation. The acute rejection rate in the gH mismatch group was significantly higher than those of the D+/R- (p=0.0051) and gH match (p=0.014) groups.
B, Kaplan-Meier curves for the cumulative probability of freedom from biopsy-proven acute rejection. The incidence of acute rejection in the gH mismatched group was significantly higher than those in the D+/R- and gH matched groups (p=0.002). Figures were modified from our recent paper (Ishibashi, Tokumoto et al. 2007).

4.4 CMV infection and disease in gH mismatch settings

Incidences of CMV infection and CMV disease were monitored in the D+/R-, gH matched and gH mismatched groups of transplant recipients (Figure 5). Antigenemia using pp65 antibodies was routinely evaluated weekly for six months after transplantation. CMV

infection was defined as the occurence of antigenemia (> 1 pp65-positive cells/300,000 leukocytes) during the monitoring period irrespective of clinical manifestations. Patients with a low level of antigenemia (1 to 9 pp65-positive cells per $3.0x10^5$ leukocytes) were monitored frequently and preemptive therapy was initiated once 10 or more pp65-positive cells per $3.0x10^5$ leukocytes were detected. Irrespective of antigenemia results, patients with CMV-related manifestations were treated immediately. The CMV-related manifestations include unexplained fever (temperature $\geq$ 38°C with no other source to account for it) for 2 or more days, arthralgia, leucopenia (leukocyte count of $\leq 4000/mm^3$), atypical lymphocytes of $\geq$ 3% or thrombocytopenia (platelet count of $\leq 100,000/mm^3$), a rise in liver enzyme level, gastrointestinal ulceration or hemorrhage, and pneumonitis (Singh, Yu et al. 1994; Tanabe, Tokumoto et al. 1997).

Sixteen of 24 recipients (67%) among the D+/R- group had CMV infection, and 13 (54%) of them developed CMV disease (Figure 5A). Among the 84 D+/R+ transplant pairings, 77 pairings were classified as gH-matched pairings or gH-mismatched pairings. During the 6-month follow up after renal transplantation, 37 (48%) of the 77 recipients in the D+/R+ group were found to be positive for CMV infection by the pp65-antigenemia assay, and 13 recipients (17%) were diagnosed with CMV disease. The incidence of CMV disease in the recipients in the D+/R- group was statistically higher than in those in the D+/R+ group (p=0.0003). When the 13 recipients in the D+/R+ group who experienced CMV disease were classified by antibody response against CMV gH, 9 (9/32, 28%) were in the gH mismatch group and 4 were in the gH matched group (4/45, 9% Figure 5 B). Consequently, CMV disease was significantly more prevalent in the gH mismatched group than in the gH matched group. The proportion of cases with CMV infection that progressed to CMV disease in the gH mismatched and gH match groups were 64% and 17%, respectively (p=0.0038, Figure 5 C).

4.5 CMV pp65 antigenemia and antibodies against gB and gH matching

We performed antigenemia assays weekly during the 6-month follow up. The medians (ranges) of the maximum number of positive cells in the recipients from the D+/R+ gH mismatched and gH matched groups were 38 (1-818) and 2 (1-142), respectively. The maximum number of pp65-positive cells differed significantly between these two groups. The medians (ranges) of the maximum number of positive cells in the recipients from the D+/R- group was 113 (1-3128), and there was no significant difference between the D+/R- group and the gH mismatched group (Ishibashi, Tokumoto et al. 2007). These results reflect the incidence of CMV disease among each group. Matching of antibody responses against strain-specific CMV gH could reduce the risk of CMV disease. The gH sequences of the CMV strains from the recipients who showed the highest level of antigenemia after transplantation were found to match the antibodies present in their donors (Ishibashi, Tokumoto et al. 2008).

In addition to gH, gB is considered to be one of the major target molecules for neutralizing antibodies as well as for cellular immune response (Gyulai, Endresz et al. 2000). We also evaluated the correlation between the maximum number of pp65-positive cells during the 6 months after transplantation and antibody responses against the gB AD2 site I epitope, as well as the matching of antibodies against gH. Seventy-seven recipients in the D+/R+ matched gH or mismatched gH groups, were classified into 4 subgroups based on the

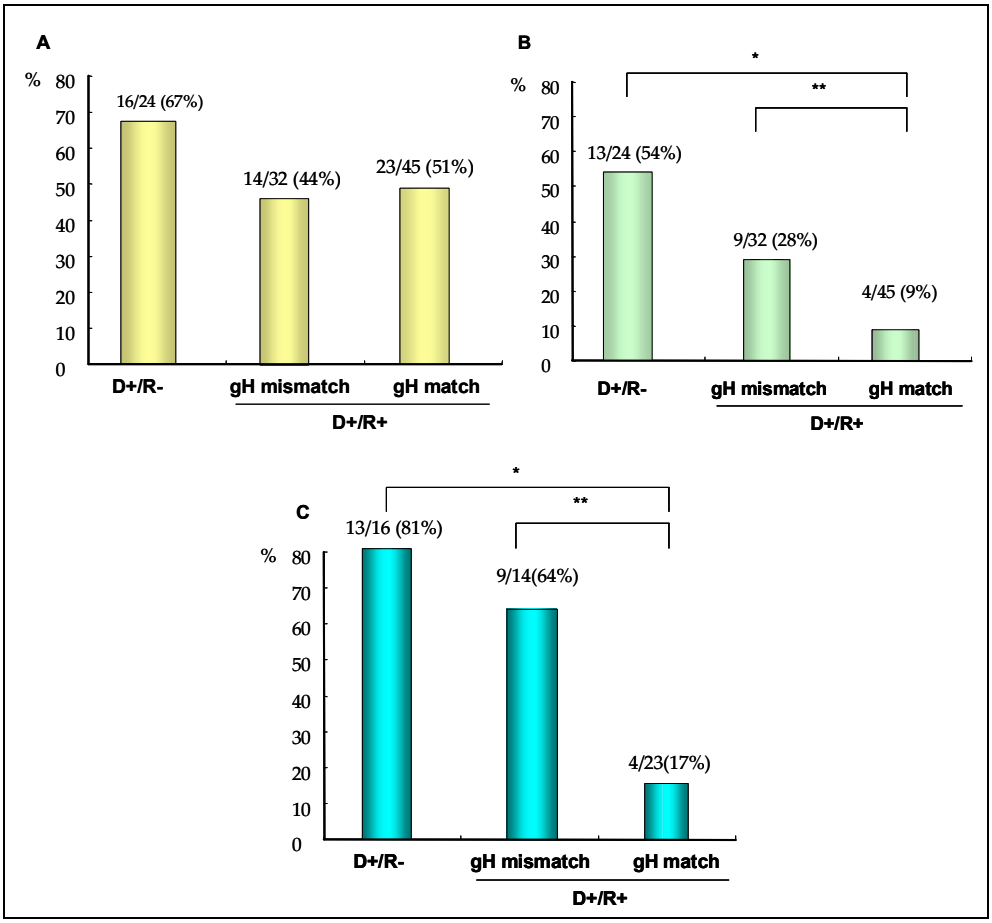

Fig. 5. CMV infection and CMV disease after renal transplantation.
A, The rate of recipients diagnosed with CMV infection. If recipients showed at least 1 pp65-positive cell during the follow up, they were diagnosed with CMV infection. There was no significant difference among the groups. B, The rate of CMV disease among the three groups. In the D+/R+ gH matched group, the rate of CMV disease was significantly lower than those in the other groups (*p=0.006, **p=0.013). C, The rate of recipients with CMV infection who progressed to CMV disease. Among the 53 recipients diagnosed with CMV infection, 26 patients progressed to CMV disease. Among them, 13 were D+/R- and 9 were gH mismatched. These rates were significantly higher than that in the gH matched group (*p=0.0001, **p=0.0038). Figures were modified from our recent paper (Ishibashi, Tokumoto et al. 2007).

combinations of antibody responses against both gH and gB (matched gH/gB-, matched gH/gB+, mismatched gH/gB-, mismatched gH/gB+). Significant differences in the maximum numbers of pp65-positive cells obtained during the 6 months in the patients with CMV infection were observed. The medians of the maximum number of positive cells were

1 (range, 1–9), 20 (range, 1-142), 26 (range 1-254), and 45 (range 2-818) for the matched gH/gB-, matched gH/gB+, mismatched gH/gB- and mismatched gH/gB+ groups, respectively (Figure 6). These differences resulted from differences in the number of patients who diagnosed with CMV disease and given preemptive therapy. Consequently, the introduction of preemptive therapy was significantly more prevalent in the subgroups lacking antibodies against gH and/or gB than in the matched gH/gB+ subgroup.

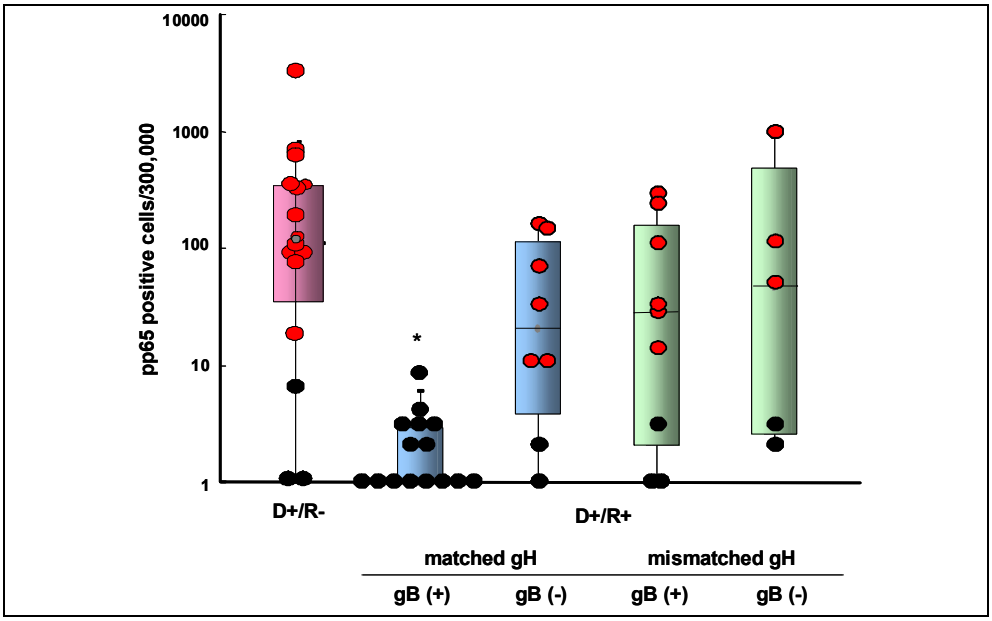

Fig. 6. Maximum number of pp65-positive cells.
Antigenemia in the 5 subgroups of transplant recipients classified based on seroimmunity against the particular epitopes of CMV gH and gB. The maximum number of pp65-positive cells during the monitoring period for each recipient with CMV infection was plotted. Each closed circle indicates one recipient. The broken bars in the box plot indicate the median of pp65-positive cells. Closed red circles indicate recipients who had given preemptive therapy for CMV disease. Figures were modified from our recent paper (Ishibashi, Tokumoto et al. 2011).

Previous studies have demonstrated that the antibody titers against AD2 measured in ELISA had a good correlation with neutralizing antibody titers (Rothe, Pepperl-Klindworth et al. 2001). However, the role of neutralizing antibodies in the control of CMV infection in transplantation recipients has been a matter of discussion (Schoppel, Schmidt et al. 1998; Volpi, Pica et al. 1999; Munoz, Gutierrez et al. 2001). Alternatively, positive anti-gB AD2 antibodies may indicate the presence of sufficient anti-CMV cellular immune responses as a consequence of the natural course of CMV infection. Among the viral proteins in CMV, gB has been identified as a potential target for CMV-specific CD8+ and CD4+ T-cell immunity (Gyulai, Endresz et al. 2000; Elkington, Walker et al. 2003). Deficiencies in the response of cytotoxic T lymphocytes specific for CMV are important in the pathogenesis of CMV disease

in immunocompromised recipients (Reusser, Riddell et al. 1991). It would be interesting to see whether the CMV specific T-cell activity against CMV-gB correlates with the outcome of our ELISA against gB AD2.

However, CMV infection was not completely prevented in spite of the presence of antibodies against matched gH and gB. A recent report on bacterial artificial chromosome-derived recombinant viruses that differed only in the expression of the gN genotype on the background of the AD169 strain showed that 30% of human sera showed strain-specific neutralization (Burkhardt, Himmelein et al. 2009). It suggested that gN constituted a major determinant for the induction of strain-specific neutralizing antibodies as well as gH and gB. The antigenic variations in a single envelope protein of CMV were shown to be sufficient to escape neutralization. It would be interesting to see the matching of antibodies against strain-specific gN in addition to the matching of gH

5. Association of CMV reinfection and acute rejection

Many reports indicate a relationship between CMV infection and allograft rejection in renal transplantation (Lautenschlager, Soots et al. 1997; Humar, Gillingham et al. 1999; McLaughlin, Wu et al. 2002; Sagedal, Nordal et al. 2002; Nett, Heisey et al. 2004). According to a prospective study of 477 consecutive renal transplant patients, CMV infection and disease are independent risk factors for clinical acute rejection (Sagedal, Nordal et al. 2002). In another study of 106 consecutive renal transplants, CMV disease, but not asymptomatic infection, was shown to be an independent risk factor for biopsy-proven acute rejection (Reischig, Jindra et al. 2006). Control of CMV infection has the potential to reduce the prevalence of acute rejection. Even one episode of acute rejection of renal allografts substantially shortens overall graft survival (Ferguson 1994), and prophylaxis could offer greater benefits compared with pre-emptive therapy. Patients provided with prophylaxis showed a significantly lower incidence of biopsy-proven acute rejection compared with those undergoing pre-emptive therapy (Reischig, Jindra et al. 2008). Prophylaxis has been shown to improve long-term graft survival in renal transplant patients (Pachl, Probert et al. 1989; Kliem, Fricke et al. 2008).

In our study, which applied pre-emptive therapy, acute rejection was observed in 32 (32%) of 101 renal transplant recipients. Among them, patients in the mismatched gH group, which indicates reinfection with a different gH strain of CMV, showed a higher rate of acute rejection (Figure 4). It is unclear why reinfection with CMV increased the risk of acute rejection while there were no significant differences in the incidence of CMV infection or disease from those in patients with a D+/R- setting (Figure 5). Considered from the perspective of immunity against CMV, escape from humoral responses, especially from antibodies against gH and/or gB, can induce a local inflammatory response through Toll-like receptors (TLRs). Inflammatory cytokine stimulation by CMV is mediated by interaction between the glycoproteins and TLR2 (Compton, Kurt-Jones et al. 2003). Neutralizing antibodies against gB and gH inhibit inflammatory cytokine responses of TLR2 to CMV infection (Boehme, Guerrero et al. 2006). However, this lack of efficient neutralizing antibodies against glycoproteins is shared by patients in a mismatched gH setting and those in a D+/R- setting. Differences in immunity against CMV between the mismatched gH and D+/R- groups could be due to the presence of memory T cells. Abate *et al.* reported that R+ recipients treated with preemptive therapy displayed a steady and constant CMV-specific

immune reconstitution with a highly heterogeneous pattern 60–360 days after transplantation, when evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay (Abate, Saldan et al. 2010). Indeed, the R- recipients treated with prophylaxis presented a very different scenario of immune reconstitution: none of the recipients analyzed showed evident immune reconstitution up to day 180 after transplantation. In this report, which analyzed 85 renal transplant recipients, 10 (IA:7, IB:3) of 70 R+ patients(14%) and 1 (IA) of 13 R- patients (8%) experienced acute rejection (Abate, Saldan et al. 2010). Another report indicated that there were no differences in the frequency of CMV-specific T-cell frequencies between patients with and without acute rejection (Nickel, Bold et al. 2009). In cardiac transplantation, recipients with early detectable levels of CMV-specific CD4 T-cells were protected from high viral loads and acute rejection (Tu, Potena et al. 2006). To date, CMV-specific cellular responses seem to be favorable to prevent adverse events after transplantation. However, IFN-γ is a proinflammatory Th1 type 1 that regulates cell-mediated responses and favors allograft rejection (Crispim, Wastowski et al. 2010; Sementilli and Franco 2010; Tellides and Pober 2007). The excessive reconstitution of IFN-γ–secreting effector cells might contribute to allograft rejection because circulating virus-specific T cells synthesize type 1 cytokines such as IFN-γ. In addition, the correlation of viral load with the development of CMV-specific T-cell responses remains unclear. We speculate that in a mismatched gH setting, a high viral load and IFN-γ from prompt cell-mediated responses induce acute rejection, whereas recipients in a matched gH setting were protected from high viral loads and those in a D+/R-setting lacked prompt cellular immune responses.

6. Conclusion

We would like to propose a working hypothesis that the mismatch of gH types, probably due to reinfection with a strain different from the original strain, is the major risk factor for CMV disease and acute rejection after renal transplantation. Pre-existing immunity against CMV glycoproteins has a critical role in the prevention of CMV infection, and it might allow recipients to avoid acute rejection. Measuring antibodies against CMV glycoproteins for both transplant donors and recipients can provide crucial information on CMV infection, particularly CMV reinfection, and the possibility of acute rejection after transplantation.

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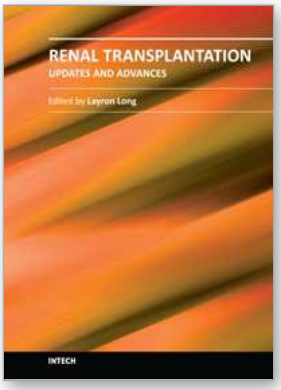

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This book presents a nice international compilation of scholarly papers and chapters which address the latest advances in renal transplant surgery. These works cover a variety of topics; the last advance and success of renal transplant science: biochemistry, immunology, molecular genetics, pharmacology - pharmacogenetics, pediatric transplant and a few rare uropathies that warrant organ replacement.

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